REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT DATE	3. DATES COVERED *(From - To)*
07-00-2002	Final	01-26-2000 to 7-15-2002

4. TITLE AND SUBTITLE
Nanode Array Sensor Microchips

5a. CONTRACT NUMBER
DAAD13-00-C-0033

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

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SBCCOM

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10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSORING/MONITORING AGENCY REPORT NUMBER

12. DISTRIBUTION AVAILABILITY STATEMENT
Distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
The funded proposal had several objectives. The first objective is to expand the fabrication and utilization of large arrays of individually addressable, nanometerscale ultramicroelectrodes (nanodes). The second objective was to expand on the current suite of immunochemical assays that can be monitored by electrochemical, visible, or fluorescent means for a wide range of chemical and biological warfare agents. Thirdly, we were to determine if various particle size (viral, spore, cell) can be detected on the ArrayChip. Lastly, limits of detection were determined for all analytes mentioned in this proposal. Attaining these technical objectives will demonstrate the commercialization of using integrated circuit sensor devices for multiplexed assays.

15. SUBJECT TERMS
Biosensors, microarrays, redox based electrochemcial detection, immunoassays, toxins.

CombiMatrix Corporation

Nanode Array Sensor Microchips

Contract #DAAD13-00-C-0033

Phase II

Final Report

July 8, 2002

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Goals and Phase II Proposal Abstract:

The funded proposal had several objectives. The first objective is to expand the fabrication and utilization of large arrays of individually addressable, nanometer-scale ultramicroelectrodes (nanodes). The second objective was to expand on the current suite of immunochemical assays that can be monitored by electrochemical, visible, or fluorescent means for a wide range of chemical and biological warfare agents. Thirdly, we were to determine if various particle size (viral, spore, cell) can be detected on the ArrayChip. Lastly, limits of detection were determined for all analytes mentioned in this proposal. Attaining these technical objectives will demonstrate the commercialization of using integrated circuit sensor devices for multiplexed assays.

The proposal was to extend the range of electrode diameters that can be fabricated on existing CombiMatrix electrode array hardware into the nanometer regime. Conventional excimer laser lithography was to be used to manufacture devices with feature sizes as small as 180 nm. CombiMatrix will combine state-of-the-art lithographic techniques with low cost CMOS device fabrication to produce analog VLSI devices with arrays of individually addressable nanodes.

The funded work was to develop methods for porting conventional immunochemical assay formats to an electrochemical as well as visible and fluorescence formats. Immunochemical assays are highly selective and are in current use for monitoring chemical and biological warfare agents. Use of electrochemical methods for detection with immunochemical assays will enable porting these techniques to arrays of microelectrodes. CombiMatrix has developed methods that enable immobilization of different assays at different electrodes in an array of individually addressable electrodes. These methods will enable electrode arrays produced by this proposal to carry numerous different immunochemical assays, which range from small molecules (saxitoxin and ricin) to viral particles, spores and cells.

Integrated CMOS sensor devices that can perform numerous simultaneous assays for the presence of a variety of chemical and biological warfare agents can fill an important role in field deployable threat assessment tools. The CMOS sensors enabled by the technologies described in this proposal

are i) small, ii) have very low power consumption characteristics, iii) perform simultaneous assays for numerous chemical and biological warfare agents, iv) have low levels of detection, v) are highly selective, and vi) are low cost.

Analytes tested

The analytes proposed to DoD as models in our assay system were: saxitoxin, ricin, α1-acid glycoprotein (AGP), phage, and Bacillus *globigii* spores (BG, as an anthrax model). Because of the difficulty in locating good antibodies against saxitoxin, we had substituted fluorescein as a simple, single epitope model for saxitoxin.

The analytes range from organic moieties, to proteins, glycoproteins, viral particles and spores. The smaller molecules contain a single epitope, whereas the larger entities more than likely contain multiple epitopes and also many copies of the same epitope. It most cases, a polyclonal antibody may be used in the assay protocol because of the increased assay performance due to the number of antigen binding sites.

For assay purposes, the fluorescein moiety is linked to a macromolecule; thus the macromolecule contains numerous fluorescein entities (6-10 units). The large protein molecules (Ricin and AGP) are extremely soluble in aqueous media. As far as we can tell, this is also true for phage where we used an anti-coat antibody (M13) for capture and detection.

Table I

Analytes Tested

Analyte	Mw	Number of Epitopes	Assay Type
Saxitoxin	299	1	Competitive
Fluorescein	332	1	Competitive
Ricin	~66,000	> 1	Sandwich
AGP	~40,000	>1	Sandwich
Phage (lamda)	[0.1 μ in diam.]	>>>1	Sandwich
BG Spores	[0.6 μ in diam.]	>>>1	Sandwich

The nature of the spore epitope is unknown, but it must be associated with a coat protein or glycoprotein. It is known that the spores tend to aggregate and thus they do not resuspend readily and settle out quite rapidly. This fact

makes the use of assays by soaking (diffusion) very difficult. In the case of a biowarfare attack, the anthrax spores would be "milled" in order to provide a very fine powder. This powder is then mixed with an aerosol (organic) material that would make the product easier to deal when in the liquid-based suspended form. Because of the nature of this problem, the media needs to be circulated or shaken so that the particles do not settle out onto non-specific areas of the chip.

Assay Formats

There are currently two assay formats that are being used in our system: [1] Sandwich and [2] competitive. Diagrams for the various formats are shown below. The sandwich assay format is the traditional and preferred format for the larger analytes. The competitive assay format is used when a small molecule contains only a single immunological epitope. In all cases, the sensitivity [dynamic range and LOD] of the sandwich assays is always better/greater than that observed for the competitive assay format. This

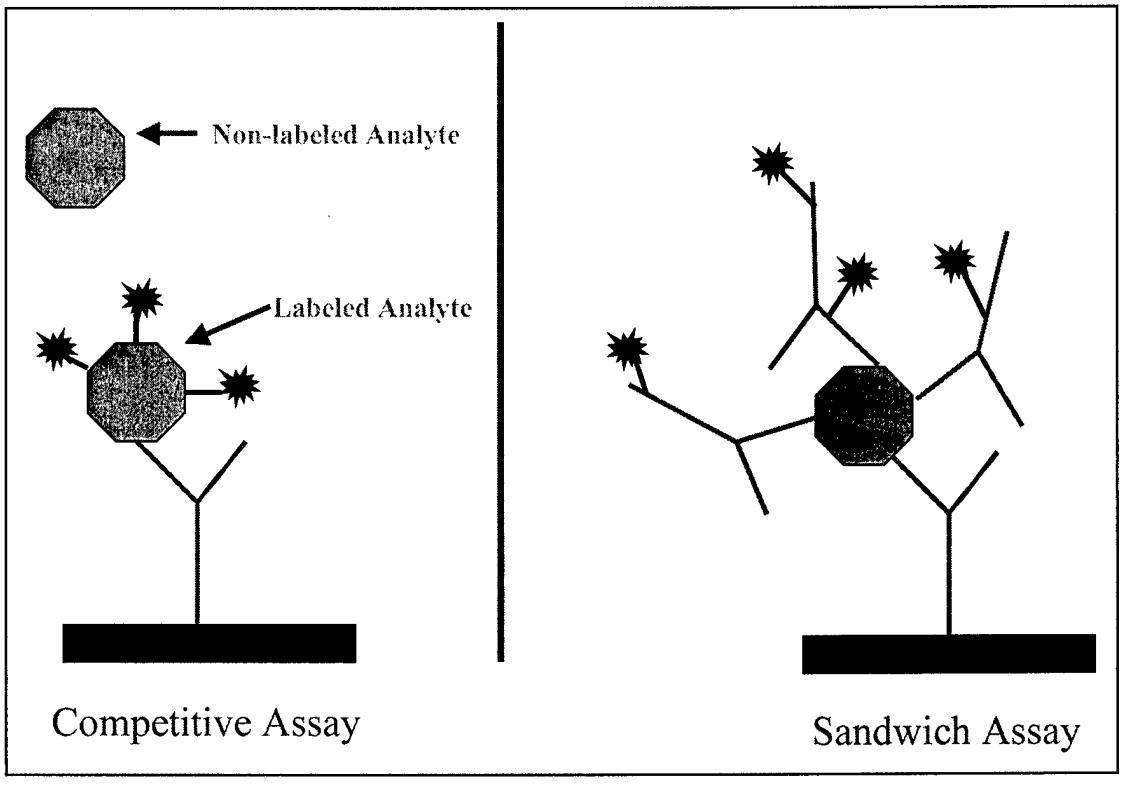


Fig. 1. Assay Formats

results from a variety of reasons, one of which is that the monoclonal antibodies used in this assay tend to have a lower affinities for the analyte than do the polyconal antibodies. Additionally, a noticeable change in signal may not be observed until 10% of the labeled material has been displaced.

The sandwich base immunoassay is composed of two antibodies: A capture antibody and detection antibody. The detection antibody may contain the detectable label or may be a point of recognition of a secondary-labeled anti-species antibody. For best results, the capture antibody is usually a monoclonal antibody derived from mouse hybridomas and the detection antibody is a polyclonal antibody (form goat, rabbit, etc.). In this fashion, the detection and capture antibodies do not compete for the same binding site. Alternatively, the same polyclonal antibody may be used for both capture and detection of the analyte. However, the LOD's may not be optimized.

Self-Assembly of antibodies and Multiplexing of the Analyte-Detection.

In Phase I of this DoD proposal we had shown that immunoassays could indeed be undertaken on the Combi-chip, but the antibodies themselves are attached to the membrane in a non-specific manner using biotin/SA complex. Because the attachment method was non-specific and it would be difficult to place multiple antibodies on the chip at one given time. Additionally, for ease of use the antibodies need to be "self assembled" (in one mixture) so time is not a factor in producing a chip that is ready for use. An antibody system that is analyte-multiplexed and self assembled, a method was necessary to develop, which would allow us to provide a finished and ready to use product.

To this end, we have developed a method called "coded affinity tag" that allows us to self assemble the antibodies on the chip in a single mixture. For this tagging method, we utilized the established Combi DNA synthesis technology to produce unique oligonucleotides at each electrode. The individual addressable electrodes along with the virtual flask technology, allows unique syntheses of 15 Mers at each electrode. For our analysis of the various analytes, we typically used 16 electrodes across the width of the chip for each analyte.

The affinity tag labeling is done as follows: a commercial vendor prepared an oligonucleotide complement to an oligonucleotide 15-Mer synthesized on the chip. The oligonucleotide either had an extender on the 5-end (close to 50 atoms), which included and SH (thiol) group at the terminus (purchased with the thiol group protected) [called the SH complement]. Alternatively, the oligomer complement could also be purchased with a 6 atom extender containing an amino group at the 3' terminus [amino complement]. To date, we have used the SH complement a vast majority of time and utilized the alternative linking method on a few occasions.

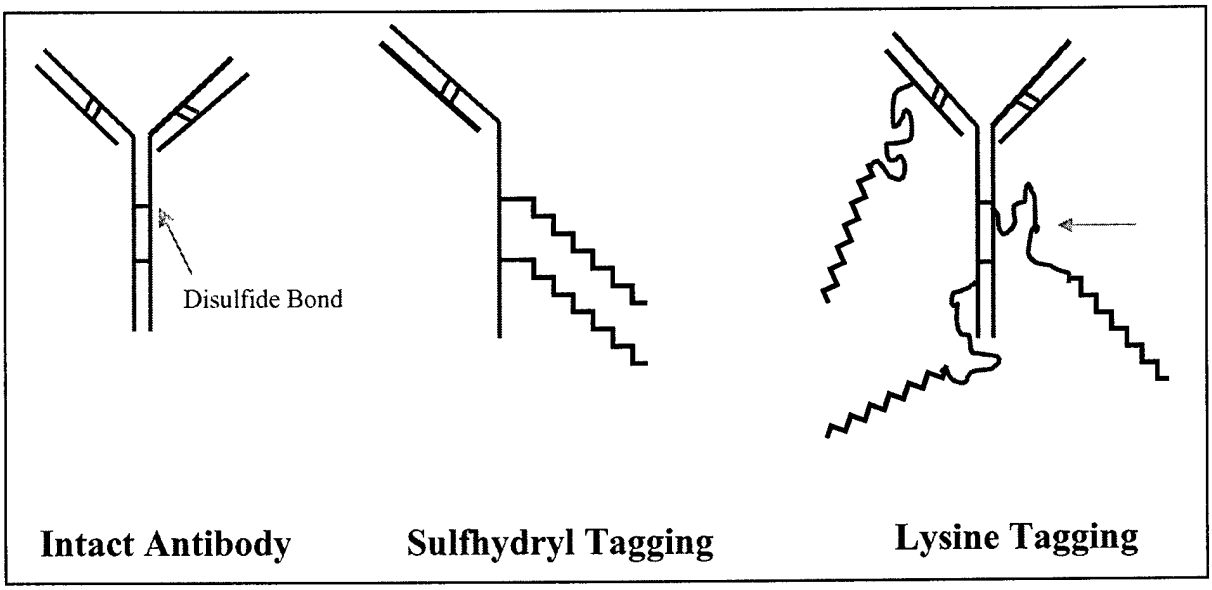


Fig. 2. Affinity Tagging of Antibodies

The complementary oligonucleotides are attached to the "capture antibodies" by the heterobifunctional chemical coupling reagent, SMCC or its sulfo derivative. Details are given in Addendum II and the reaction diagrams are given below. In both cases, two functional groups must be present: a free thio and a free amino group. The first method uses the free ε-amino groups found on lysyl residues of the protein (there should be multiple residues) and the free thiol groups of the oligonucleotide. Alternatively, the amino group may be used for attachment on the amino complement and a thiol group on the antibody. Since the antibody contains no free thiol group, the protein must be selectively reduced to make

available two free thiol groups found on the heavy chains of the antibody (see diagram).

Fluorescence Based Detection as a Control

Traditional detection methods for any immunoassay are absorbance based, where an enzyme catalyzes the turnover of a substrate into a product that is color producing in either the solution phase or a colored precipitate. In the latter stages, the substrate could be a compound that fluoresces when converted to product by an enzyme. This recent procedure has been found to be increasingly sensitive.

Along these lines, an immunoassay was developed whereby the detection antibody in the sandwich complex contains a fluorophore (such as fluorescein, Texas Red, Cy5) that can be detected. If the assay follows the competitive assay format, one of the competing analytes must contain a fluororphore.

This fluorescence based format is very traditional and thus was used to confirm and corroborate the self assembly of antibodies as well as to establish the function/viability of immunosandwich assays that were formed.

Electrochemical Detection

We have found that fluorescence-based immunoassays do not provide a large dynamic range (2 logs at best) nor do they render an LOD that meets current market acceptable detection limits.

An alternative to the fluorescence-based is electrochemical detection-based enzyme-amplified immunoassays. In this case a "redox signal" is produced when the enzyme converts substrate to product. Many times more than often, the reaction requires a mediator or a co-reactant that can be monitored at the electrode surface. The enzyme itself alone does not need to be considered a redox enzyme, if the product conversion requires the release/consumption of electrons. The generation or consumption of electrons can be monitored at the electrode in question as either a current difference (in nanoamps or subnanoamps) or as a potential difference (nV).

Table II

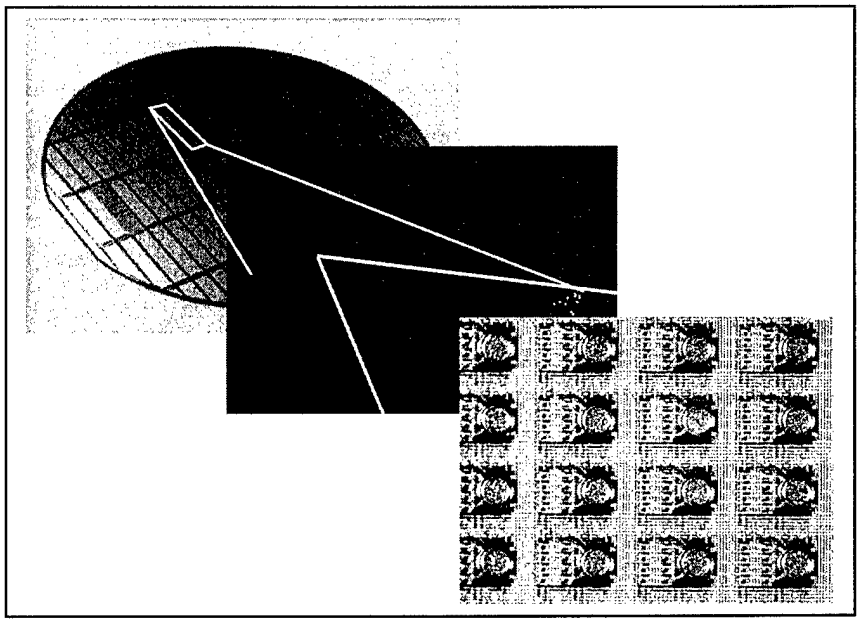
Redox Enzymes

Enzyme	Mw	Redox	pH Opt.	Sub/Prod. Sol	Stability	Med.
β-Gal	550K (4)	N	7.5	low	excellent	Y
Glu-Ox	400K (4)	Y	7.0	high	excellent	Y
HRP	31K	Y	5.5	high	good	N
Laccase	50K	Y	5.5	high	good	N

Each enzyme has it's pros and cons, but we found that horseradish peroxidase and laccase best fit our needs. Considerations for each enzyme substrate system were: [1] enzyme stability; [2] optimum pH range in which the enzyme functioned; [3] substrate stability; [4] substrate solubility; and [5] product solubility. They are much smaller than β-galactosidase and glucose oxidase and the substrate turnover is considerably higher. Their only liabilities are their solution stabilities. The enzyme reactions for the conversion of substrate to product reactions are given in addendum. Clearly, the redox products that are highly soluble and that do not require electron-shuttling intermediary, are preferred. Thus, enzymes such as HRP and laccase are perfect for this study.

Electrochemical Chip

The current ship in use at CombiMatrix is the CME9608I. This is an old design with antiquated on-board storage and electronics. However, each electrode is individually addressable which makes it usable for both DNA syntheses and for e-chem detection. The current chip also lends itself to modifications, such as the etching to produce 5-micron vias within the 100-



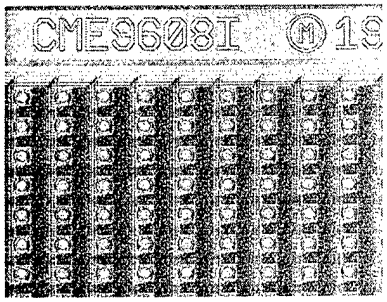


Figure 3

micron diameter electrode region. However, the hardware and software are such that the e-chem detection (electrode sample) can only be done individually and one at a time. This makes the sample rate very slow and in some cases, well after the "end point" of the experiment has been reached.

The ability to sample multiple electrodes (8 in this case) concomitantly will allow better std. curve data as the concentrations of analyte will be determined based upon enzymatic kinetics rather than a nebulous endpoint. Additionally, rapid analysis of all of the electrodes at once provides a quick and convenient method for analyzing and comparing data that is not skewed by time; this is often encountered when using endpoint data across a wide range of analyte concentrations.

E-Chem Instrument Prototype

In order to make this system more user friendly, we will be providing DoD a prototype of an electrochemical detection system that has the software

required to accumulate and process signal data from our chip. The unit needs to be "semi automated (or at least be in a position to automate down the line) and have the ability to move liquids through the chamber via a robotic system. In this way, reagents can be pumped into a tightly sealed reaction/hybridization/e-chem detection chamber. It is critical that the system be allowed to contain limited volumes (80 microliters or less) and that the chamber can be flushed with buffer or specific reagents added.

RESULTS

Fig 4. Shows a CAD drawing of the prototype with a robotic system. The e-chem unit is visible as the robotic arm can be used to introduce samples and reagents to the unit. Within this unit will be our assembled E-chem unit as depicted in Fig. 5. The unit is barely the size of two palms and can be transported by hand. For the moment, it is attached to a desktop computer and a Keithly voltage/current regulator/detection unit. This can be miniaturized at a later date with the introduction of a smaller current/voltage detection system and laptop computer.

Prototype Fluid Interface/Automated Sample Handling/HTS

An important design decision was the fluidics interface that was chosen for the prototype. A conventional pipette tip provides the widest range of options for the user. The sample collection and initial processing for this prototype must be performed either by third party equipment (*e.g.*; the FOX environmental sampling system) or by the user. A pipette tip interface also allows the unit to be used with conventional laboratory robot systems. A laboratory robot system is a rapid way to introduce automated sample and fluidic handling tasks. Figure 4 illustrates an electrochemical assay unit mounted on a small laboratory robot.

Waste management is an important issue for sample that may contain chemical and biological threat agents. The prototype will have a disposable fluid waste container. The user can dispose of the contaminated sample without risk of exposure. Alternatively, the user can keep the waste container to recover the sample at some future time.

Within this unit is that chip mounting system that contains a peltier controlled temperature control unit. Additionally, an electronic board is mounted in the back, which controls and the processes (Figs. 5 & 6). The chip is attached to a PGA package of mounting and electronic addressing. The front part of the chip contains the electronically active immunochemistry portion for assays and detection. The chip is enclosed in a sealed cell, so that chemistries may be performed (hybridization, antigen addition, enzyme substrate addition, antibody addition as chip washing) and the chip checked for immunochemical results. The cell housing and be removed, cleaned, and then subsequently used on other e-chem chip assemblies. For future endeavors, the chip may be sectored and then used with micro fluidic setup for a higher throughput of biological samples.

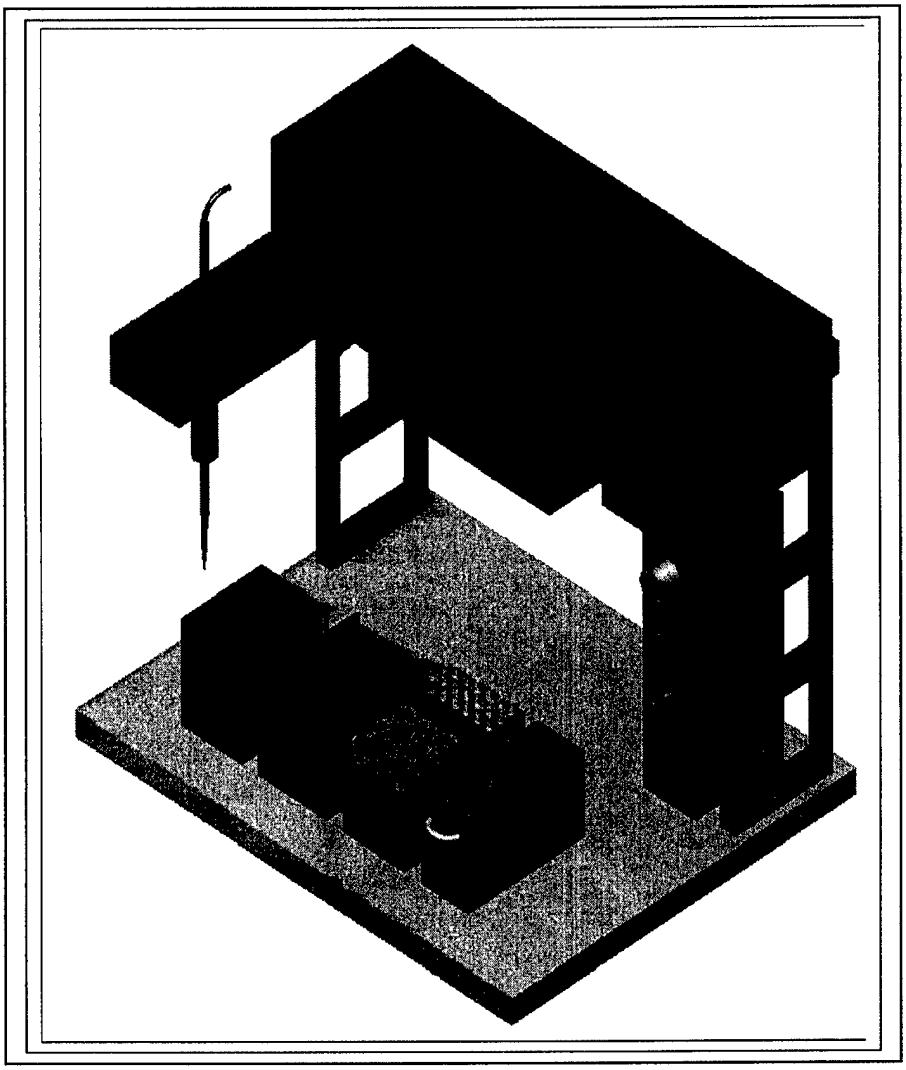
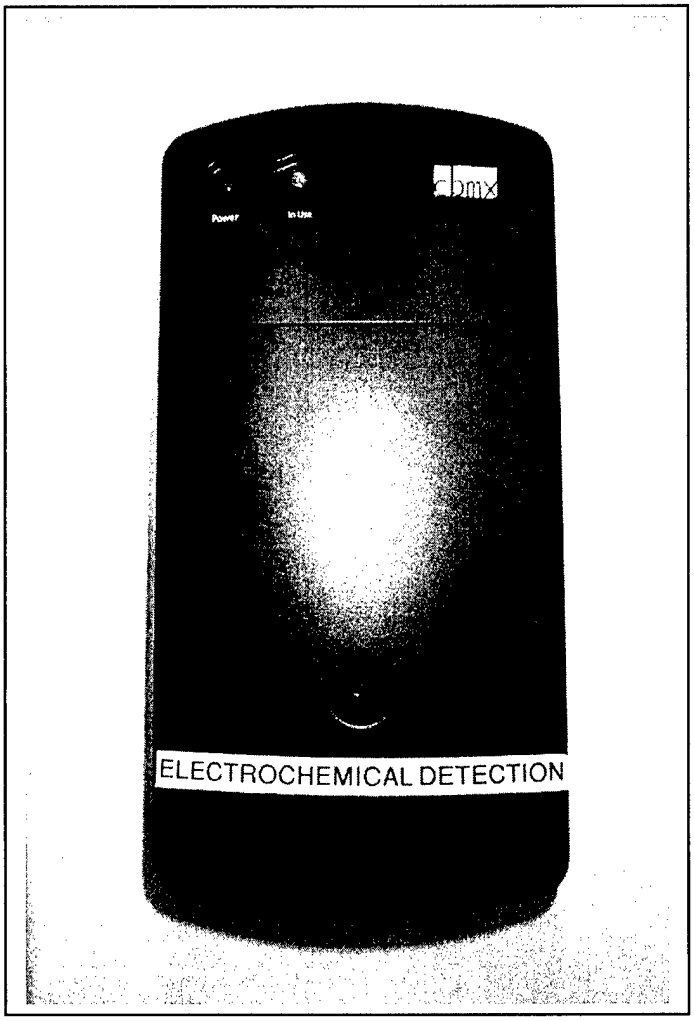


Fig. 4. E-Chem Robotic System

The unit contains a reaction/e-chem chamber that is depicted in Fig. 7. Our recent results indicate that the volume safely can be brought down to at least 50 microliters in this microchamber. Fluid flow considerations have become the gating design issue at this juncture. Studies of sample chamber geometries that allow smaller volumes are still on going.



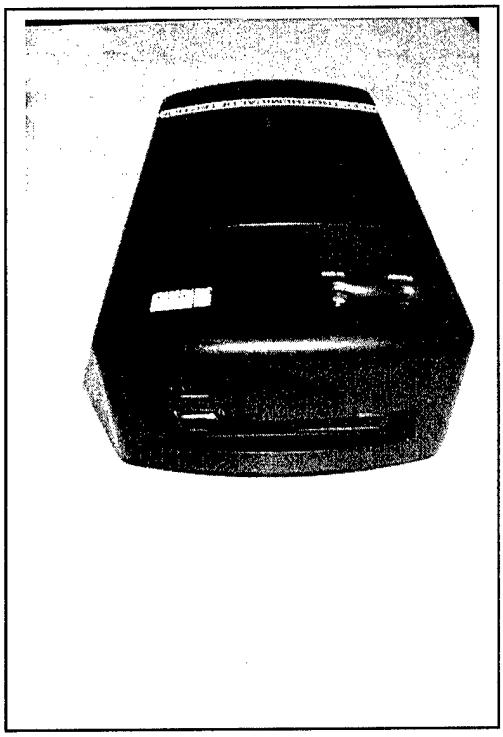


Fig. 5. E-Sense System

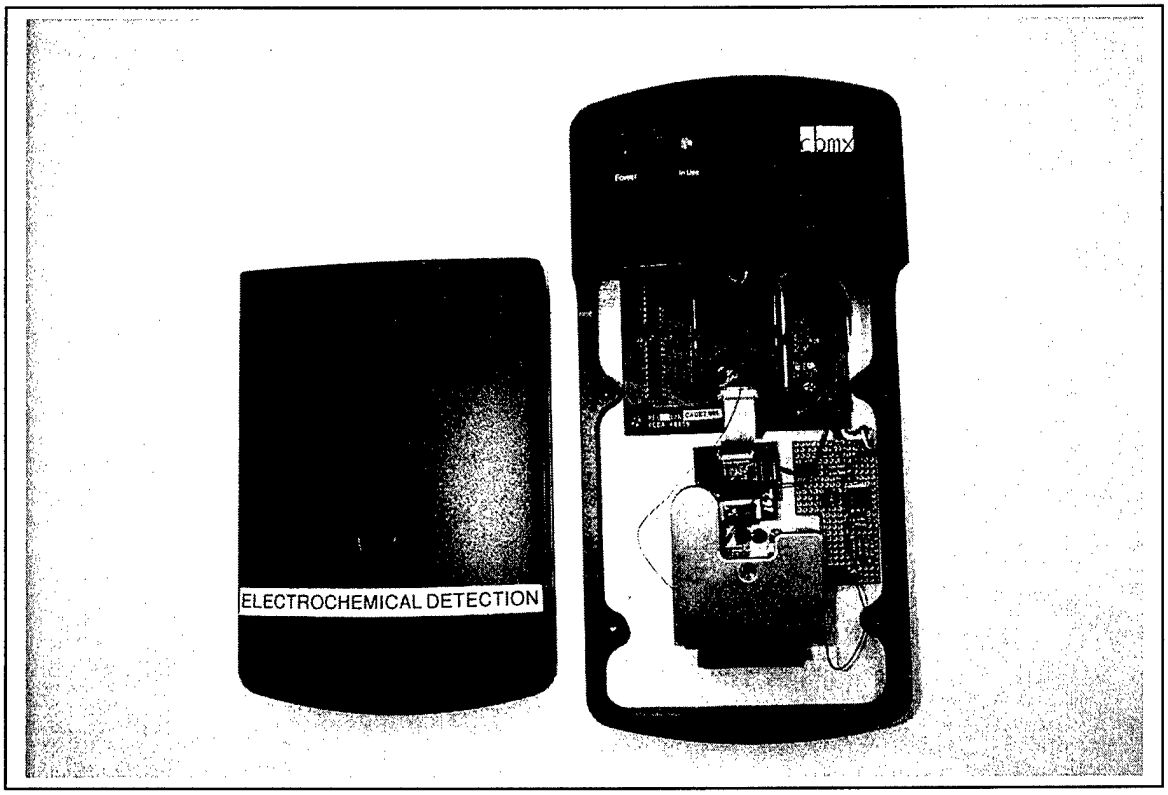


Fig. 6. E-Sense System

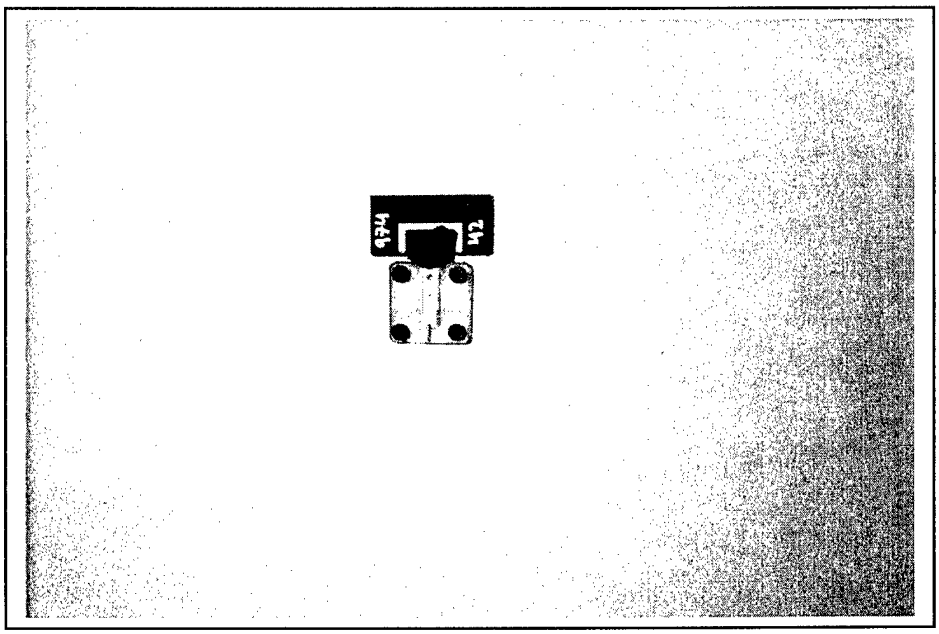


Fig. 7. E-Sense Flow Chamber

Assays Utilizing Fluorescence Based Detection

The fluorescent-based detection protocol was originally envisioned to establish that the antibodies did indeed self-assembling on the chip surface; immunoassay-based detection of analytes was possible through the formation of an immunosandwich when a second, labeled was added.

The downside to this is that a fluorescence label must be attached to a number of protein moieties: [1] the secondary antibody of the sandwich; [2] a secondary antibody that recognizes the sandwich complex; [3] labeled streptavidin. In many instances the fluorescence tag has little affect on the structure or function of the tagged protein.

To demonstrate that the "Affinity Tag System" methodology is viable for multiple antibody immobilization, a mouse antibody and a rabbit antibody were labeled with specific and unique affinity tags. A CombiMatrix ArrayChip was then produced that was labeled with complementary Anchor Tags that were specific for these two antibodies. The solution containing both rabbit and mouse antibodies with Affinity Tags were incubated with a chip containing complementary Anchor Tags for one hour at 40°C. The chip was washed and then immersed in a solution containing Texas Red labeled goat anti-mouse and fluorescein labeled goat anti-rabbit antibodies for 30 minutes. The immunoassay scheme for this experiment is shown in Fig. 1. Fig. 8 shows the results from this experiment, using both a Texas Red filter and a fluorescein filter. This figure clearly shows that the "Affinity Tagged" mouse IgG migrated to the upper section of the chip and could be detected with the TR-labeled goat anti-mouse antibody. The "Affinity Tagged" rabbit antibody bound specifically to the membrane above the electrodes in the lower section of the chip, and it was specifically detected with fluorescein-labeled goat anti-rabbit antibody.

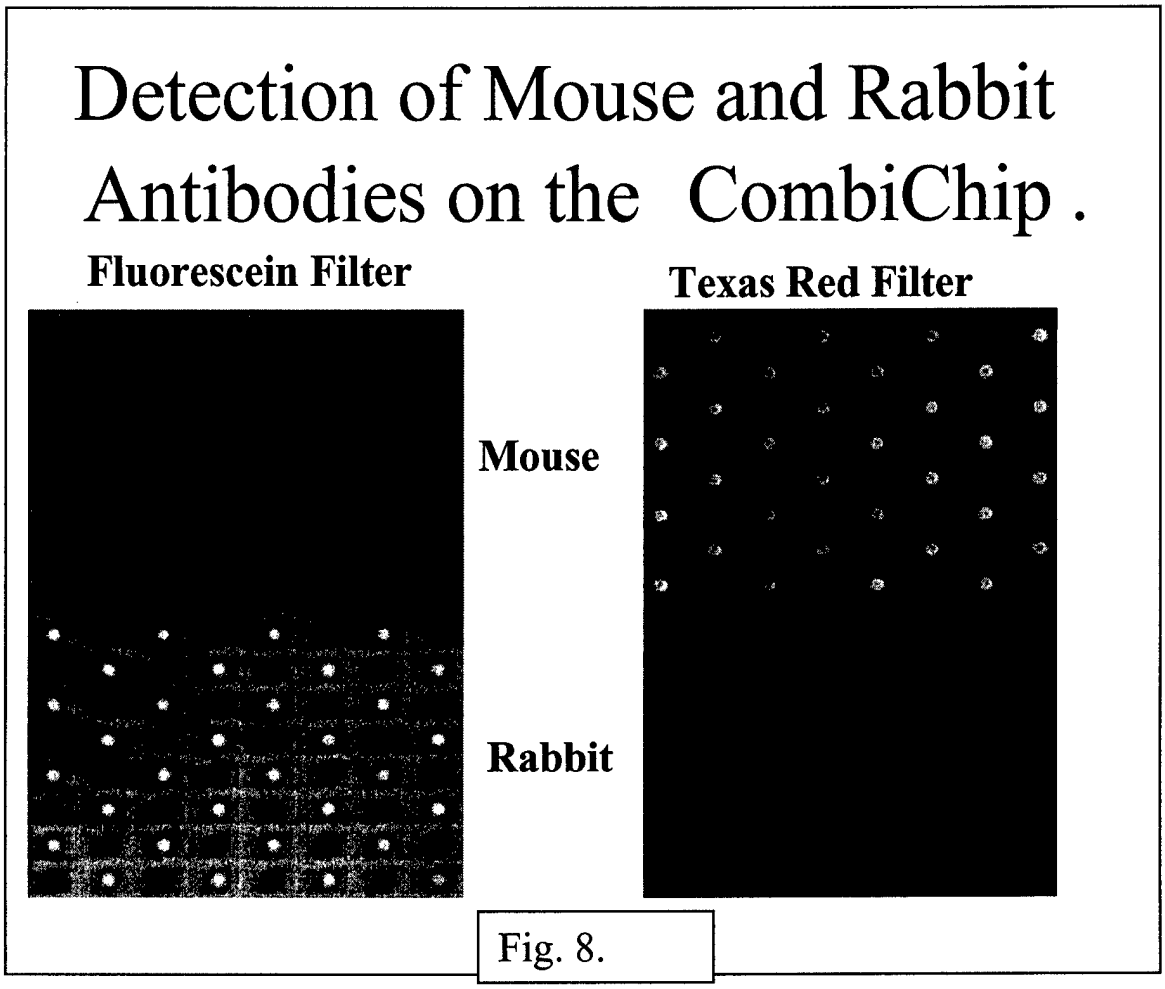


Fig. 8.

This experiment established the fact that we could self assemble antibodies (or any proteins) on the chip surface. But can the system work for immunoassays? The results for the sandwich-based assays are shown in Fig. 9. In each case the detection is fluorescence based and using a sandwich assays format.

Immunodetection of Various Analytes on the CombiMatrix Biochip

An antibody specific for each analyte is captured in an individual lane on the chip based upon the a pre-attached "coded affinity tag". Lane 1 is always reserved for our internal reference . Note that we always incubate a chip with antibodies for all analytes at one time.

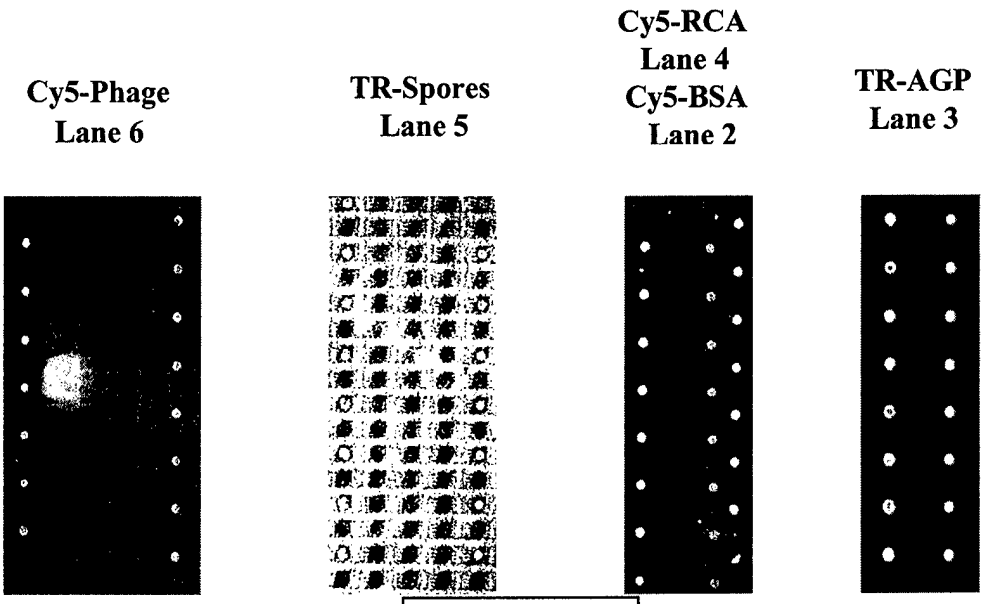


Fig. 9.

The result from a competitive assay format is shown in Fig. 10. The assay curve was determined for the fluorescein-analyte system. The results are given in Fig. 5. The data for this figure were obtained using a 1 μM solution of the capture fluorophore. The fluoresceinated competitive inhibitor was added at the concentrations shown on the x-axis. For each data point the capture fluorophore and F-inhibitor were pre-incubated with the anti-fluorescein antibody before the complex was captured on the CombiMatrix biochip. The resulting fluorescence is given on the y-axis. If the capture fluorophore was first placed onto the biochip and the anti-fluorescein antibody pre-incubated only with the F-inhibitor, the competitive inhibition curve is shifted to right (higher inhibitor concentrations). Clearly the limits of detection are more difficult to extract when using the competitive assay format.

Competitive Immunoassay System for F-BSA and F-BSA-Cy5 Using an Anti-Fluorescein Antibody Bound to the CombiMatrix Biochip.

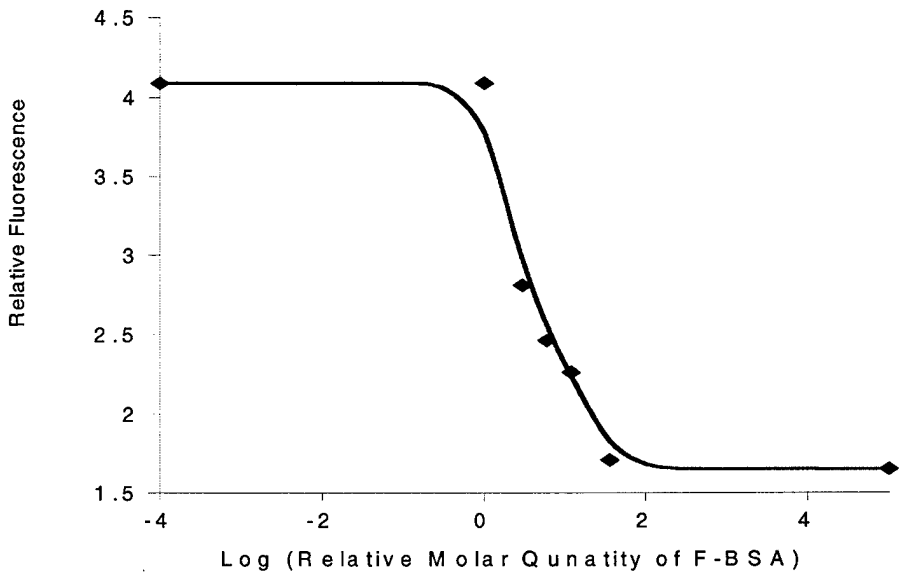


Fig. 10.

Another use of this chip and format is the detection of post translationally modified proteins or in the case of modified or altered cells or spores. To show that we can indeed determine molecular changes on the surface of a protein, we modified α1-AGP with neuraminidase, a glycosidase that removes terminal neuramninc acid from several oligosaccharide units found on AGP (to form asialo-AGP). The resulting enzyme action results in the display of a terminal b-galactose units which can be detected with β-galactose specific lectin, ricin.

Fig. 11 shows our results from our work with AGP and AAGP. Both AGP and AAGP can be detected using an antibody that is specific for protein epitope. However, when the sialic acid is removed and ricin used in the detection process, only the AAGP can be detected, as would be expected.

Detection of Post Translationally Modified Proteins

Sandwich Assay for AGP and AAGP Based upon Protein Epitopes

Sandwich Assay for AGP and AAGP Based upon Protein and Carbohydrate Epitopes.

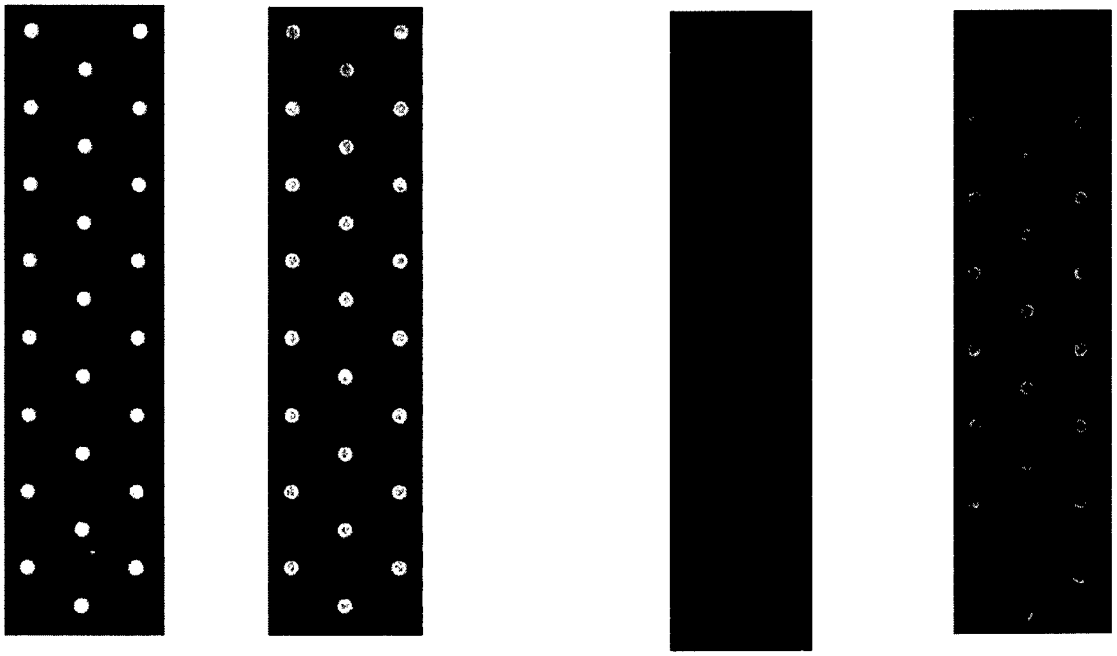


Fig. 11.

Enzyme Amplified Redox Chemistry (Electrochemical Detection)

Since our initial phase I studies, we have investigated numerous additional enzyme systems that may be used for our enzyme amplified electrochemical

detection on the CombiMatrix biochip. The enzymes investigated over the past three months include, β-galactosidase, glucose oxidase, horseradish peroxidase and laccase. The first three enzymes have been studied in the most detail to date and the enzymatic reactions utilized are given below.

Oligonucleotide detection

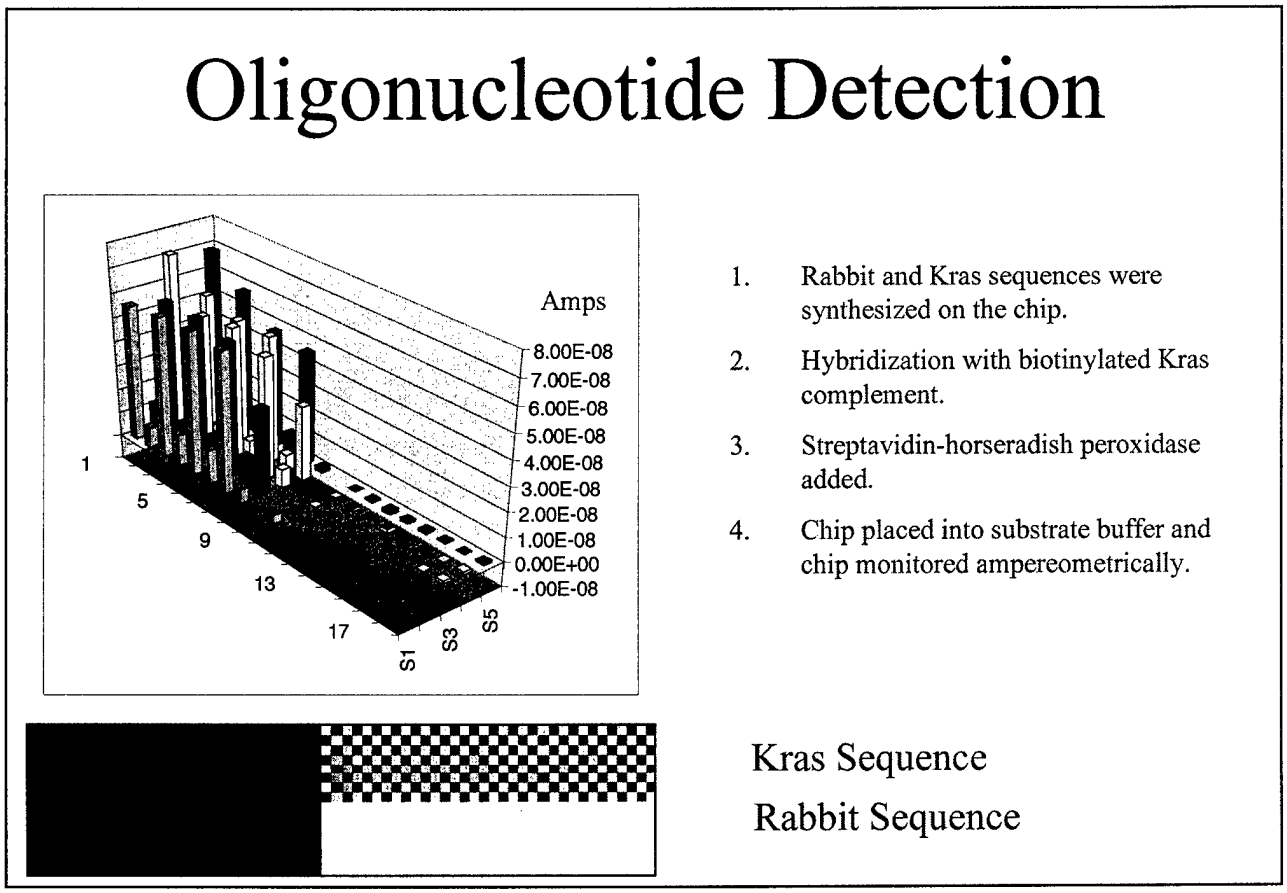


Fig. 12.

A simple e-chem detection scheme would be two different oligonucleotides synthesized on the chip (15-mers). The chip is then hybridized with 15-mer complement to the Kras sequence that contained a biotin molecule. When the chip was subsequently incubated with streptadivin-HRP, a e-chem signal (in nano-amps) could be detected at the appropriate electrodes (Fig. 12).

Simultaneous detection of two analytes by e-chem

In a simple sandwich assay format e-chem detection experiment, we chose AGP and phage as our test analytes. These are both sandwich based assays and the detection antibody contained a biotin labeled antibody. The chip was finally incubated with streptavidin-HRP to provide the signal generation. Fig. 13 shows the results.

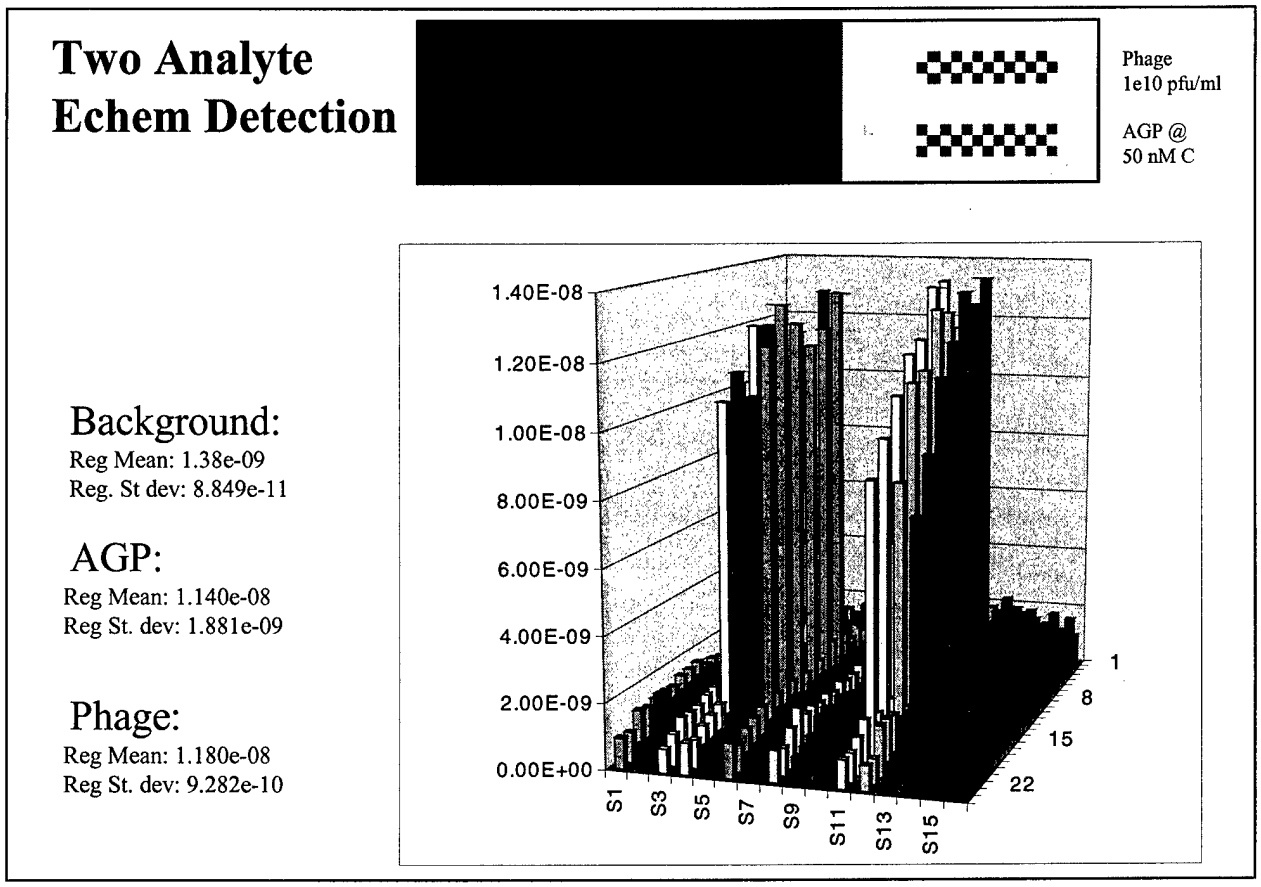


Fig. 13.

The data for figure 13 were collected with new software that was recently developed at CombiMatrix and with some hardware changes to the e-sense system.

Simultaneous detection of multiple analytes by the e-chem method

Development of assay protocols for detecting all of the required analytes simultaneously was the prime directive in the phase II contract. A global assay protocol and standard operation procedures are given in the addenda to this report. The results for the simultaneous detection of all five analytes are shown in Fig. 15. The extremely intense signal for F-BSA masks the excellent results that we obtained for all the other analytes. To show that we can detect each analyte individually by the e-chem method, please refer to quarterly report #4.

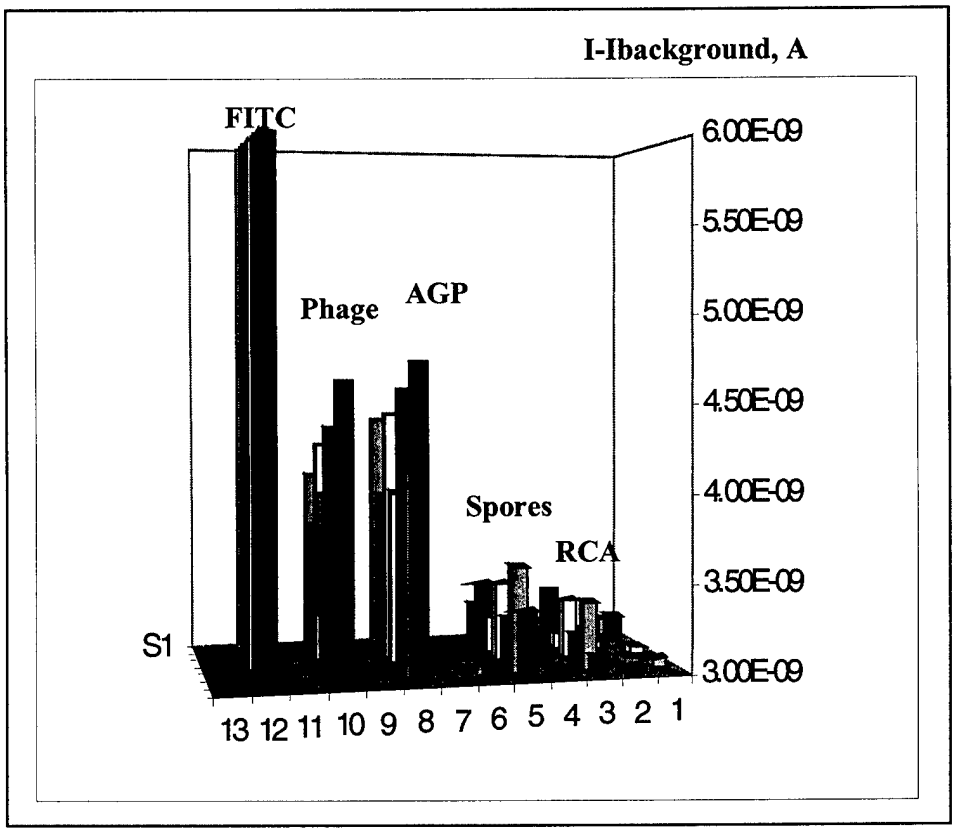


Fig. 15.

E-chem Limits of detection and dynamic range

Fig. 16 shows some standard curves developed for Ricin and AGP. Note that the curves are rather linear over the analyte concentration range investigated. The y-axis is defined in terms of nano-amps while the x-axis is represented in terms of log analyte concentration. The 0-point is defined as 1 picogram per mL. The LOD for the system has been determined for two analytes: AGP and RCA. In both cases the best results were obtained when using the HRP-SA polymer and the LODs were in the range of 5 pg/mL and 300 pg/mL for AGP and Ricin, respectively. Since the chip is immersed in about 0.5 mL, this gives 80 amol (and 2,500 amol for ricin) that can be bound to the antibodies on the chip.

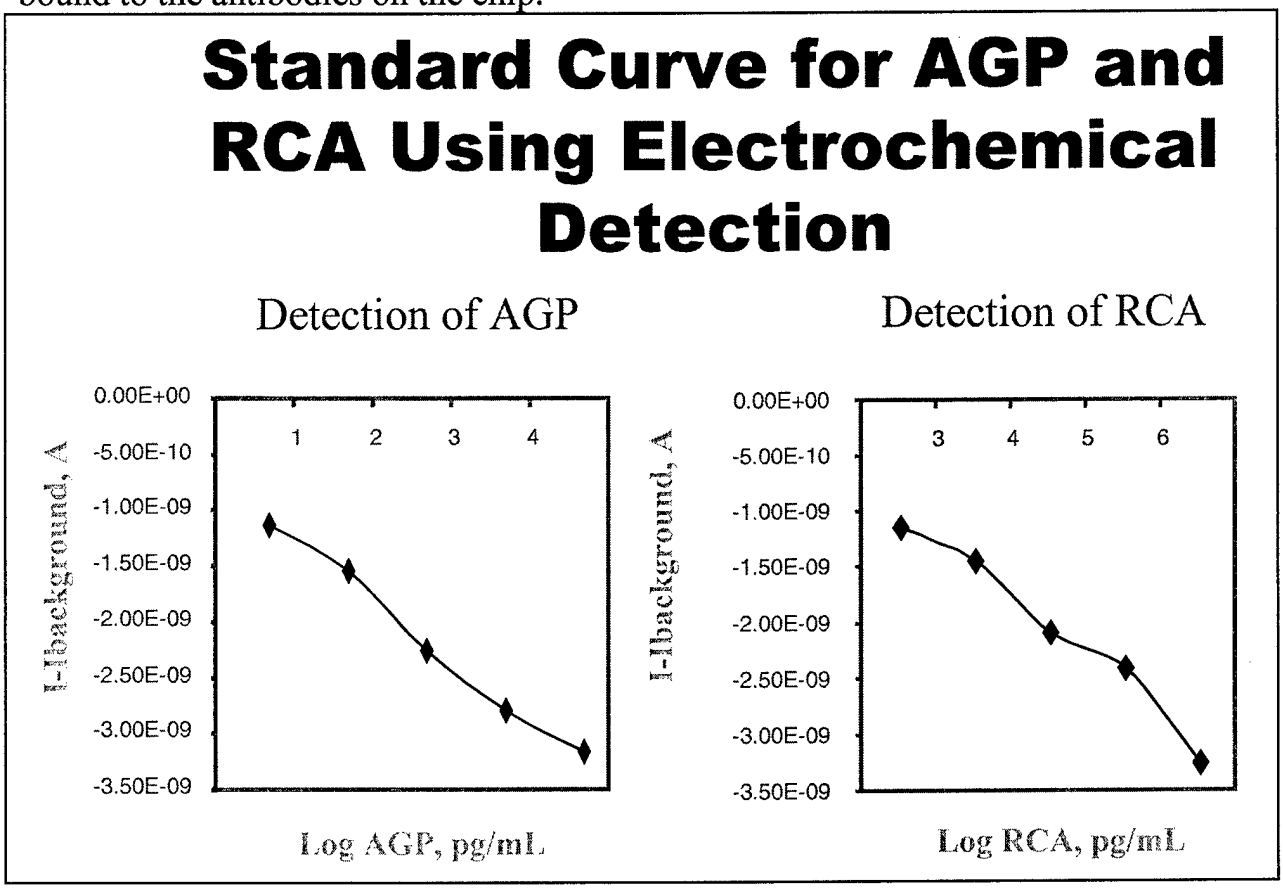


Fig. 16

Considering that in most cases we use 16 spots/electrodes for analyses and all the solution analyte were bound each electrode, then each electrode could contain a maximum of less than one amole of analyte. The detection of 1 amol in ½ mL volume is quite impressive and better than most analytical systems currently on the market (Fig. 17).

Dynamic Range and Limits of Detection for Various Analytes

1. **AGP: 5 pg/mL. 80 amol in ½ ml volume.**
2. **RCA: 300 pg/ml. 2.5 fmol in ½ ml volume**

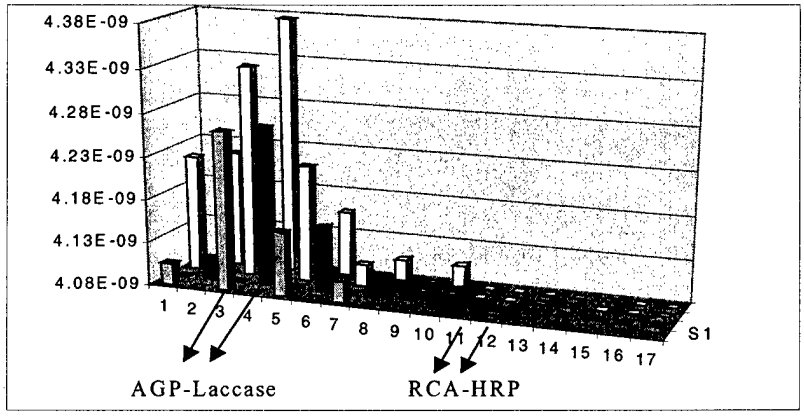
Dynamic Detection Range is 4 logs

Fig. 17

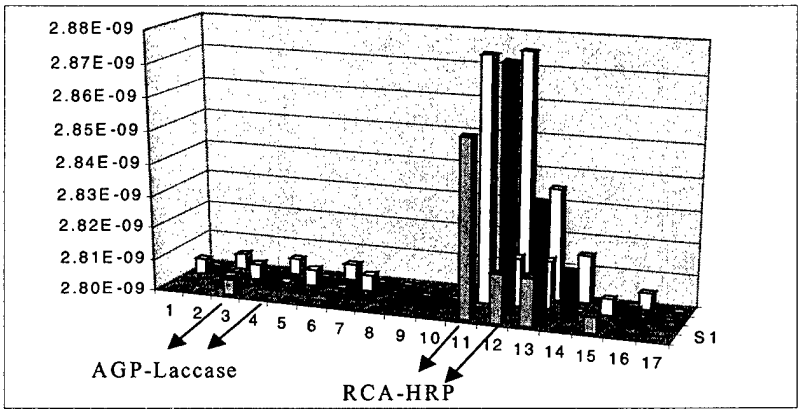
Dual Enzyme Detection System

In the last report, we demonstrated a "dual enzyme system" that allowed us to detect two analytes (ricin and human α1-acid glycoprotein) captured on the chip surface using different enzyme labels. This was accomplished via the direct conjugation of either a laccase enzyme or horseradish peroxidase enzyme directly to the signal antibody. In both cases, analyte-specific polyclonal antibodies were tagged with a capture oligonucleotide and self-assembled on the chip surface. Analytes were sequestered by capture antibodies on the biochip and the enzyme-labeled signal antibody was allowed to bind and form an immunosandwich.

Our initial experiments, detailed in report #6, required a subtractive protocol to distinguish signals from the two different enzyme labels. We have now established a protocol that allows a clean discrimination of the signals from each of the two label enzymes. Conditions have been established in this protocol such that the voltage setting and the pH is the same for both of the enzyme labels. However, the enzyme substrates differ and the order of reagent addition in this protocol is crucial. The signal from the laccase enzyme labels in measured first using catechol as a substrate. After measuring the laccase signal, the biochip is washed and the second substrate solution is added to measure the signal from the HRP enzyme label. OPD is used as the substrate for the enzyme HRP. Signals from the two enzyme labels are clearly distinguished using this protocol as illustrated in Figure 18.



Substrates: Catechol atmospheric O_2



Substrates: OPD-H_2O_2

Figure 18. Two step measuring protocol for discriminating signals from laccase and HRP reporter enzymes. Here AGP secondary antibody was labeled with laccase and ricin secondary was labeled with HRP.

Nanoelectrode development

To show that the Combichip can be further miniaturized into a "nanoelectrdoe technology", we created several chips that contain "vias" within the 100 micron electrode of our 1K chip (Fig. 19). In this case the small electrodes (5 microns in diameter) are generated in the area within the 100 micron region. The microelectrodes were covered with our proprietary 3-dimensional membrane system. Then the DNA sysnthesis was performed at nanoelectrodes to produce a 15 Mer oligonucleotide. The 15 Mer was then hybridized with a complementary 15 Mer DNA strand containing a Texas Red label at the tail. The results obtained from a microelectrode chip using an epifluorescent microscope are shown below in Fig 2. The data indicate that CombiMatrix is close to producing "nanoelectrodes" that can be used in future studies.

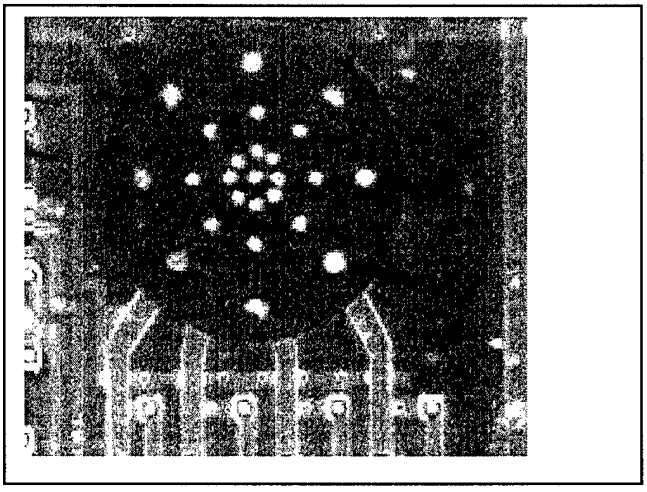


Fig. 19. Nanoelectrodes of 5 micron diameter. These are 5 micron diameter spots shown in a ring like structure within the 100 micron space. The outer ring contains microelectrodes that are 10 microns in

diameter.

Future work

For future work, we are in the process of obtaining (designed by SemQuest, Inc.) a new chip that would allow better individual charge storage, faster addressability, and multi-electrode data download. The electrode data can be downloaded from 16 electrodes simultaneously; a entire 1K array can be in less than 100 microseconds. Thus, data arrays can be collected so that real time "enzyme kinetic" data can be collected form each electrode. Specification details of this chip was given in report #7 and repeated in addenda.

Software requirements document

The software requirements document (SRD) is a companion document to the hardware specification that is being developed to guide software development and functionality. The software that is currently in use will serve as the kernel for a software package that will be delivered with the prototype unit. This software package will include all functional components that are necessary for a user, but will simplify the user interface and mask much of the deep level functionality of the software that most users do not need to access. Full access to deep level functionality will be retained for service personnel.

ADDENDA

1. Affinity Tagging of Antibodies
2. Biotin and Fluorescent-Moiety Labeling of Antibodies
3. Assembly of Primary Antibodies on the Chip
4. Sandwich Immunoassay Procedures
5. E-chem Assay Protocols
6. Prototype Hardware Specifications
7. Redox Chemistries for Enzymic Reactions

Affinity Tagging of Antibodies

Materials

Oligo with free thiol group on 5' end (attached by 18 C linker) (Operon)

10x Phosphate Buffered Saline (PBS)

Dithiothreitol 1mg/ml (DTT) (Sigma #20290)

Succinimidyl-4-(N-Maleinidomethyl) (SMCC) 1mg/ml (Pierce # 22360)

Dimethylforamide (DMF) (Sigma)

1xPBS 1mM EDTA

P30 columns (Bio Rad #732-6202)

P6 columns (Bio Rad #732-6200)

Poly-HRP 80-SA (Research Diagnostics # RDI-PHR80-SA)

EZ link Sulfo-NHS-LC-LC-Biotin (Pierce #21338)

Protocol

Thiol modification of Oligos:

1) 10 µl 5' thiol modified oligo (use 1mM stock)
2) 30 µl 1 10X PBS
3) 5 µl DTT 1mg/ml
4) Allow reaction to incubate at RT for 4 hours, or overnight at 4° C.
5) Equilibrate 2 P6 columns with 1X PBS-1mM EDTA, 3X-500 µl.
6) Apply the mixture to the prepped columns and collect.

Crosslinker addition to antibody:

(Molar ratio should be 5-10 SMCC to Ab)

* if Ab is not suspended in 1X PBS, than buffer reaction in 1X PBS

1) Dissolve SMCC in DMF to a final concentration of 1mg/ml (3mM)
2) 1.2 µl SMCC (1 mg/ml) (.07mM in 51.2ul)

3) 50 µl desired Ab (apx. 50 µg) (6.5 µM final in 51.2µl)
4) Incubate at RT for 2 hours
5) Equilibrate 2 P6 columns with 1X PBS-1mM EDTA, 3X- 500 µl
6) Apply the mixture to the prepped columns and collect.

Conjugation Reaction:

1) Combine reactions in tube and incubate for 2 hours at RT
2) Equilibrate 2 P30 columns with 1X PBS-1mM EDTA, 3X-500µl
3) Apply the mixture to the prepped columns and collect.
4) Aliquot and freeze for storage

Coupling of Antibody (and other Proteins) with Biotin or Fluorescein/Texas Red/ Cy5

Pierce's standard protocol (for item #21338) or Molecular Devices' procedure for their Threshold Immunoassay System can be used for the various labelings. The labeling procedure is the same whether NHS ester or the isocyano derivative is of the specific moiety is used. In essence, the labeling material which is usually sealed and kept away from moisture) is dissolved in fresh, dry DMF to approximately 10 mg/mL. Small aliquots of this solution (3-10 μL) are added to an aqueous, buffered solution of protein (pH 7.5-8) so that the DMF content of the final solution is less than 10% and the molar coupling ratio (moiety to protein) was between 10 and 20/1.

The reactants were mixed under vigorous vortexing and allowed to react for 1 hr. at room temp. Follwoign this the reaction was desalted (or placed into an appropriate buffer) by passing the reaction mixture (no greater than 75 μL) through a phosphate buffer, pre-equilibrated P-6 spin column.

Primary Ab Self Assembling on The Chip (Chip Preparation)

Primary Ab tagged to the certain oligomer is suspended in 2x PBS containing 0.05% Tween 20 (2x PBST) at an approximate concentration of 1-5 μg/ml. The chip is incubated with the oligo tagged Ab for 1 h at 40 C^{o}. The chip is then washed with 4 ml of 2x PBST.

Sandwich Immunoassay Procedure

1. Analytes of different concentration are suspended in 2x PBST. The analyte(s) containing solution is introduced to the chip. The chip is incubated with the analyte(s) containing solution for 1h at room temperature. The chip is then washed with 4 mls of 2x PBST.

2. Secondary Ab linked to either HRP or biotin suspended in 2x PBST is then introduced to the chip. Incubate for 1hr at room temperature. The chip is then washed with 4mls of 2x PBST.

3. **Proceed to E-CHEM detection if Ab-HRP conjugate is used*
HRP-streptavidin conjugate (1 μg/ml) in 2x PBST is introduced to the chip. The chip is incubated with HRP-streptavidin conjugate solution for 1h at room temperature. The chip is then washed with 4mls of 2x PBST and electrochemical detection is then performed.

Electrochemical detection

The electrochemical detection is performed in 50 mM citrate-phosphate buffer pH 5.0 that contains 0.2 M NaCl. Substrates used are 1 mM o-Phenylene diamine (OPD) and 0.003% hydrogen peroxide. The measurement duration is 0.5 sec, the delay is 0.3 sec. Voltage set between the chip and a counter electrode is -0.3 V. The resulting signal corresponds to the average current of the analyte bound region minus an average background current taken from a designated 'background area'.

Prototype Hardware specification

Top Level Specifications

Parameter	Specification	Comments
Intended Use	• Instrument for producing arrays of immobilized proteins and measuring appropriate assay parameters with electrochemical methods	
System Components	• Electrochemical Measurement Instrument • Desktop PC (as specified), cables • Control software • Chips (format TBD) • Goal: Fluidics automation	
Number of chips	• Prototype instrument will support one chip	• May be expanded to support more chips simultaneously in future designs
Number of assay sites per chip	• Must look at five analytes	• Number of assay sites determined by assay requirements.
Automation	• Prototype instrument will require manual assay protocols	• Full automation may be supported in future designs
Electrochemical measurement	• Depends on chip format. • Goal: < one second per assay site for serial measurements	• Parallel measurements will be much faster but require new echem chip design
System Reproducibility	• tbd after evaluating the error budget	• Define the experiment and evaluation for measuring the system reproducibility
Sensitivity	• Goal: 500 spores • Goal: lectin 50 pg • Goal: AGP 10 pg • Goal: phage 10^7 CFUs • Goal: FITC competitive assay 10^{-12} moles	• The sensitivity may be limited by the assay • The sensitivity needs to be established in the presence of all analytes
Dynamic Range	• Goal: > three orders of magnitude	• The dynamic range may be limited by the assay

Environmental

Parameter	Specification	Comments
Operating Conditions	• Meets GLP safety requirements	
Laboratory Environment	• only power supply required • no water, drain, gas, air pressure	
Chemical Resistance	• Resistance against all materials used • Resistance against standard cleaning solutions (alcohol and soap water based)	

Service and Installation

Parameter	Specification	Comments
Installation at customer site	• Goal: installation time is overnite, done by a service technician	
Access for service	• Preferred from instrument front	
Covers and housings	• Removable for service without influence on instrument main functions and adjustments • Easy access to all service relevant parts without disassembling larger assemblies	
Service tools, screws	• No special tooling required • Preferred screw types allen with cylinder head for all service relevant parts	
Preventive Maintenance	• Goal: max. 1 preventive maintenance per year by service technician • Goal: max . 3 preventive maintenance per year by user	

Operation

Parameter	Specification	Comments
Run start checks	• Check of all error sensors • Electrical integrity of chip	
Power up behavior	• Correct self start-up and initialization from any possible previous instrument status • No negative interference when switched on before / after PC switched on	
Sensor Concept	• Integrated circuit functionality	• Additional sensor instrumentation may be required for fluidics system
Light exposure	• No significant light during echem measurements	• specify light flux

Electrics

Parameter	Specification	Comments
Instrument Control	• External desktop PC (1 PCI slot used) • minimum PC configuration tbd • operating system tbd • RS232	• Depends on fluidics control requirement
Power Requirements	• 100-240 Volt +/- 15%; 50-6Hz • Goal: battery powered (12V) • no external range switching	
System Warm-up-time	• 20 min warm up time from power on to fulfill specifications • <5 min from standby	

Mechanics

Parameter	Specification	Comments
Package size and content	• Max 800 mm in one dimension (standard door width) • All instrument parts, manual, accessories, cables, etc in one package • External PC is supplied/packed separately	
System Dimensions	• Desktop instrument, max. footprint 400mm x 600 mm, but as small as possible • Man-portable instrument max volume 0.2 cubic meters	• This system is not automated. System size for automated unit may be larger.

Fluidics

Parameter	Specification	Comments
Fluid delivery system	• Manual delivery. • Goal: automated delivery of wash and measuring solutions	• Future designs will allow automation or adaption for laboratory robots.
Sample Introduction	• Manual • Volume tbd	• Must be sterile and confined
Waste Collection	• On-board • Goal: disposable	• Must be sterile and isolated and disposable
Total Volume per run	• Goal: <1ml	• Assume 50 microliter sample chamber volume and 15 microliter dead volume
Fluid oscillation	• Oscillation rate and volume tbd	• May be required for mixing in some chip packaging configurations

Parameter	Specification	Comments
Fluid recirculation	• Total volume tbd • Flow rate tbd	
Liquid Interfaces to chip	• pipette or syringe	• May need customized interface to different sample collection configurations

Temperature Control

Parameter	Specification	Comments
Heating/Cooling Concept	• Ambient temperature • Goal: Peltier Element with heat sink with fan	
Temperature Sensor	• pre calibrated by manufacturer	
Temp. Homogeneity (Within Chip)	• Goal: Range: 1K (temperature difference between coldest an hottest spot on the chip)	
Temp. Range:	• 20° - 45°C	
Temperature Accuracy	• Goal: ±0.5K , maximum <±1K	
Temp. Stability:	• Goal: ±0.25K over 0.5h	
Ramping time	• heating: goal >0.2K/s, acceptable 0.1K/s • cooling: goal >0.1K/s, acceptable 0.05 K/s	• rates are the mean of ramps between 45°C to 65°C (for ramp up) and 45°C to 20°C (for ramp down)

Echem

Parameter	Specification	Comments
Potential Range	• Potential difference 2V	• Note there is no reference electrode in this spec
Current Range	• 10 picoampere to 100 nanoampere	• This is a per electrode spec
Counter electrode	• External counter • Counter is an integral part of sample chamber • Material tbd (Au, Pt, ITO)	• May require separate chamber for counter electrode
Electrical source and measurement	• External to chip • Computer controlled	
Chip addressing	• Prototype will be serial addressing • Goal: parallel addressing	

User Interface

Parameter	Specification	Comments
On / Off light	• Green: Constant light when power is on.	

Parameter	Specification	Comments
Error Light	• Red: Constant light when Error occurred	
Power Switching	• Main Power Switch and fuse on instrument	

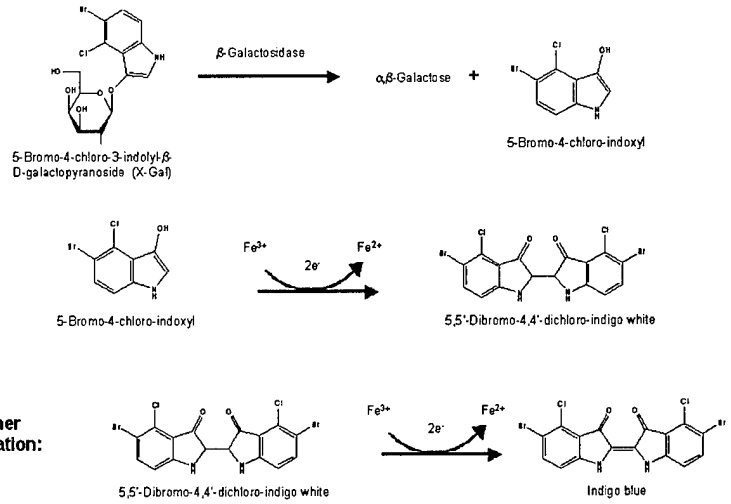
Software

Parameter	Specification	Comments
General SW Specs	• See Separate Software Requirement Document (SRD)	
Service Access	• Interface for Service SW: TBD	
Error Handling	• Error Detection and Measures done by Software	

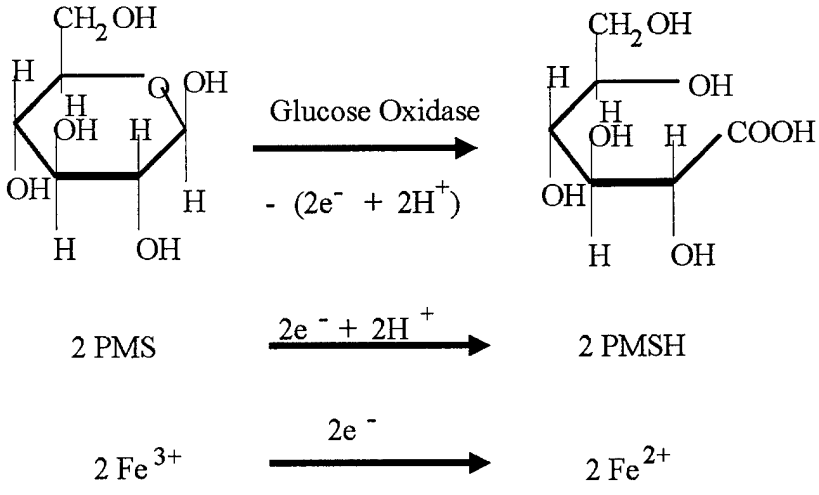
Miscellaneous

Parameter	Specification	Comments
Test Bed Configuration	• Designed for mounting on conventional laboratory robot for semiautomatic fluid handling	

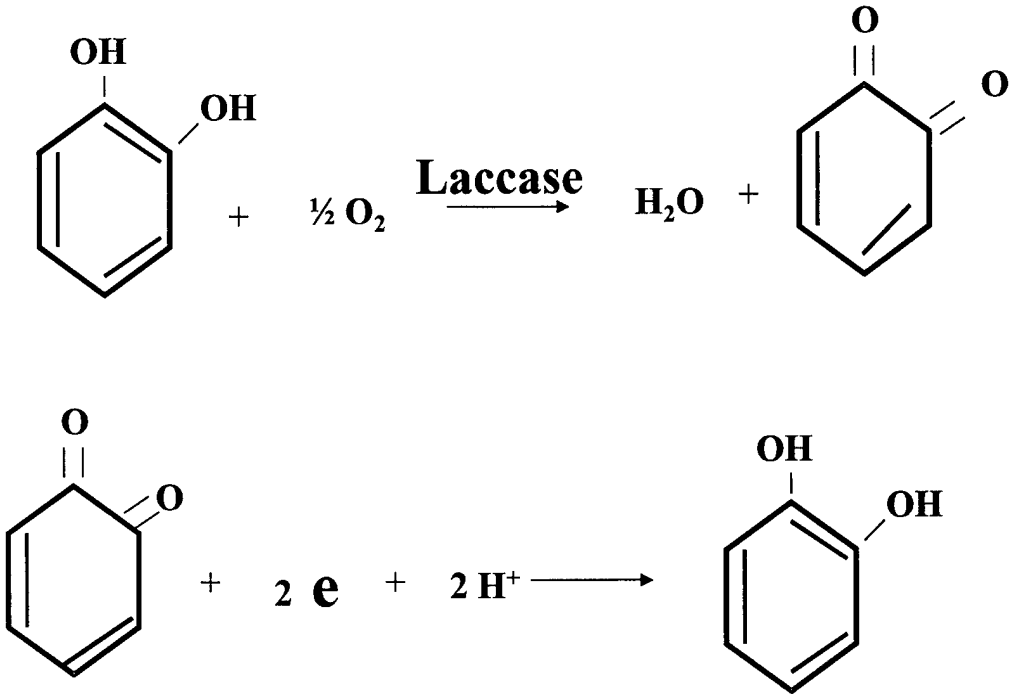
β-Galactosidase Reaction Scheme

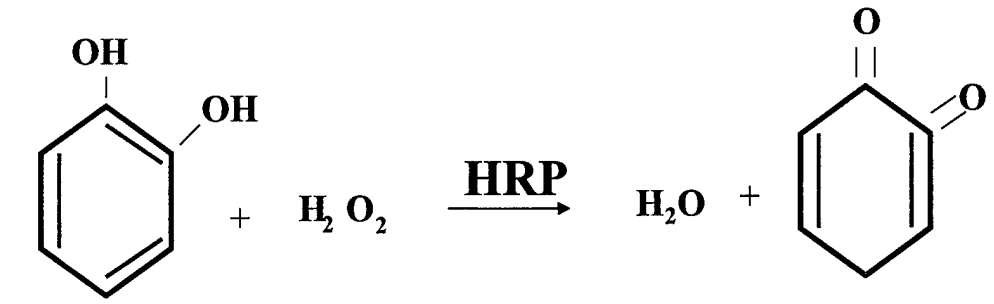


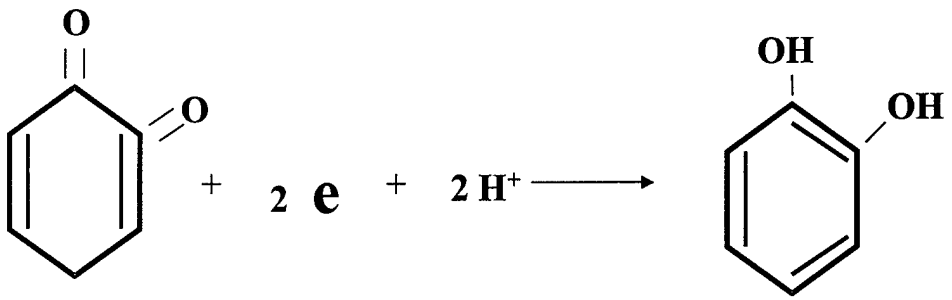
Glucose Oxidase Reaction Scheme



PMS = 5-methyl-phenazinium methyl sulfate


OH
OH
+ ½ O2
Laccase
H2O +
O
O
O
O
+ 2 e + 2 H+
OH
OH


OH
OH
+
H_2O_2
HRP
H_2O
+
O
O


O
O
+
2 e
+
2 H^+
OH
OH